Ancrod and Fibrin Formation
Perspectives on Mechanisms of Action

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Background and Purpose—Ancrod, derived from Malayan pit viper venom, has been tested as ischemic stroke treatment in clinical trials with inconsistent results. We studied the actions of ancrod on fibrinolysis pathways in patient plasma samples and endothelial cell culture systems.

Methods—We analyzed fibrinogen levels during the first 6 hours of ancrod infusion in patients entered in the Stroke Treatment with Ancrod Trial. For the in vitro study, human brain microvascular endothelial cells incubated with plasminogen or with human brain microvascular endothelial cell-conditioned medium were co-incubated with ancrod and fibrinogen under normal or oxygen-glucose deprivation conditions over 6 hours.

Results—Fibrinogen levels decreased both in vivo and in vitro. Ancrod generated fibrinopeptide A, caused visible clot formation, and reduced levels of tissue-type plasminogen activator antigen in the human brain microvascular endothelial cell system and in a cell-free system with conditioned media.

Conclusions—The in vitro results indicate that ancrod causes local fibrin formation and secondary depletion of tissue-type plasminogen activator by binding to fibrin clot. Ancrod-induced fibrin formation could result in cerebral microvascular occlusion and may explain the suboptimal clinical effects of ancrod in human stroke trials. (Stroke. 2011;42:3277-3280.)

Key Words: ancrod ■ defibrinogenation ■ fibrinogen ■ fibrinolysis ■ ischemic stroke

Ancrod has long been viewed as a potential treatment for acute ischemic stroke.1–6 Derived from the venom of the Malayan pit viper Calloselasma rhodostoma, ancrod possesses a serine protease that cleaves fibrinopeptide A (FPA) from fibrinogen.6–9 This fibrinogenolytic effect underlies ancrod’s potential clinical benefit, which would be based on limited clot propagation, reduced plasma viscosity, improved microcirculatory flow, and activation of endogenous fibrinolysis.6–9

However, results of ancrod in clinical trials of ischemic stroke have not been uniformly successful. Whereas the Stroke Treatment with Ancrod Trial (STAT) showed a favorable benefit–risk profile for patients with ischemic stroke treated within 3 hours of stroke onset,2 the European Stroke Treatment with Ancrod Trial (ESTAT) and the Ancrod Stroke Program (ASP) showed no benefit in functional outcome for patients given ancrod within 6 hours of stroke onset.3,4 To shed light on these disappointing findings, we studied the effects of ancrod on fibrinolysis by analysis of plasma samples from STAT patients and by in vitro studies with human brain microvascular endothelial cells (HBMVEC) including conditions of oxygen-glucose deprivation as an in vitro ischemia model.

Materials and Methods

Clinical Samples
We analyzed citrated blood obtained at baseline, 3, and 6 hours after starting ancrod administration in ancrod-treated STAT2 patients in whom local fibrinogen concentrations had been measured with photo-optical instruments based on the Clauss method10 (N=189, excluding 46 ancrod-treated subjects with fibrinogen measured with other instruments and 13 subjects with incomplete measurements). The study was approved by the Institutional Review Board at each participating hospital and written informed consent was obtained from all patients or their representatives.

Cell Culture and Reagents
HBMVECs were maintained and identified as previously described.11 Dulbecco modified eagle medium (Invitrogen Corporation, Carlsbad, CA) without glucose was used for oxygen-glucose deprivation experiments. Human fibrinogen was from Enzyme Research Labs (South Bend, IN), human plasminogen was from Sigma (St Louis, MO), and ancrod (72 IU/mL) was provided by Neurobiological Technologies, Inc (NTI, Emeryville, CA). The control was the ancrod excipient (NTI).

Experimental Design
In vitro experiments were performed with confluent monolayers of HBMVEC with plasminogen (0.2 mg/mL) and with ancrod (0.004 IU/mL, comparable to that measured in patients after 3 hours of
ancrod infusion) and/or fibrinogen (300 mg/dL) or appropriate control. Oxygen-glucose deprivation experiments were performed in a humidified chamber filled with 2% O2 and 5% CO2. After incubation at 37°C for 6 hours, conditioned medium was aliquoted and stored at −80°C. For cell-free tissue-type plasminogen activator (tPA) depletion studies, HBMVECs were grown to confluence and then incubated with M131 medium containing fibrinogen. After 6 hours, conditioned medium was collected and further incubated with or without ancrod for another 6 hours.

**Assays**

Plasminogen, tPA, urokinase plasminogen activator, plasminogen activator inhibitor-1, and FPA antigens were determined by enzyme immunoassay (American Diagnostica, Greenwich, CT). Fibrinogen antigen was measured by immunoassay (Diapharma, West Chester, OH).

**Statistical Analysis**
Statistical analysis was performed using paired t tests (for the clinical analysis) and analysis of variance with Tukey test. A probability value of <0.05 was considered statistically significant.

**Results**

Mean fibrinogen concentration in plasma of patients receiving ancrod decreased from 358 mg/dL at baseline to 274 mg/dL (77% of baseline, P<0.0001) at 3 hours and to 121 mg/dL (34% of baseline, P<0.0001) at 6 hours (Figure 1). After incubation of ancrod plus fibrinogen with HBMVEC in vitro, fibrin clot was present at 6 hours (Figure 2); clot was not formed with fibrinogen alone or ancrod alone. Fibrinogen levels in conditioned medium of ancrod-treated HBMVEC decreased to 91 mg/dL after 6 hours, 31% of that present using fibrinogen without ancrod (Figure 3A; P<0.0001). FPA concentration was 8.7 ng/mL with ancrod (P<0.0001) compared with negligible levels in cells treated with fibrinogen without ancrod (Figure 3B).

Ancrod reduced the level of tPA antigen in fibrinogen-enriched HBMVEC-conditioned medium to 4.0 ng/mL compared with 7.4 to 8.4 ng/mL under 3 control conditions (P<0.0001 versus ancrod alone, fibrinogen alone, and neither; Figure 3C). To analyze the possibility that ancrod may have reduced tPA release by HBMVEC, we collected media (containing fibrinogen) conditioned 6 hours by HBMVEC; we then incubated the conditioned media with ancrod for an additional 6 hours without HBMVEC. Under these conditions, clot was formed and tPA levels decreased to 1.5 ng/mL compared with 5.4 ng/mL (P<0.0001) in control (Figure 3D). These findings indicated that the decline in tPA levels was not dependent on the presence of endothelial cells.

Fibrinogen and ancrod treatment did not induce a significant decrease in plasminogen antigen levels when compared with fibrinogen alone. Ancrod induced no significant change in levels of plasminogen activator inhibitor-1 or urokinase plasminogen activator antigen. Under oxygen-glucose deprivation conditions, ancrod produced similar but generally milder effects with regard to FPA generation and tPA depletion (data not shown).

**Discussion**
We demonstrated ancrod-induced decline in fibrinogen levels in vivo and in vitro along with in vitro generation of FPA (released from fibrinogen), decline in tPA antigen levels, and production of fibrin clot. Although ancrod-induced fibrin generation is well known, the fibrin produced has usually been understood to be non-crosslinked, soluble, readily degraded, and rapidly removed from the circulation. Ancrod-induced insoluble fibrin has been previously described in a plasma-based in vitro system using ancrod at a concentration at least 2 to 3 orders of magnitude higher than was used in the current study. For the present work, we used a concentration of ancrod comparable to that used in stroke clinical trials (D. E. Levy, unpublished observations). The rapid decline of fibrinogen levels, release of FPA, and generation of insoluble fibrin clot are consistent with what is encountered in disseminated intravascular coagulation. Fibrin generation consequent to ancrod use may not be inconsequential and could contribute to cerebral microvascular occlusion.
Levels of tPA were substantially reduced when HBMVEC were incubated with ancrod and fibrinogen. In the cell-free system, tPA antigen showed a 73% decline in conditioned media, comparable to that in the presence of cells (approximately 50% depletion). This indicates that soluble tPA was depleted in the presence of clot formation and that tPA was likely bound to clot, resulting in low levels of soluble tPA in conditioned media. Prior in vivo work showed no change in levels of circulating tPA with ancrod treatment, but levels of tPA in the systemic circulation may not reflect actions in the brain vasculature and microcirculation.

It is tempting to assume that fibrin clot generation observed in our cell culture system is analogous to what occurs in vivo. However, the relationship between endothelial cell surface area and fibrin clot will likely differ substantially between in vitro and in vivo systems, the fibrinolytic capacity of our cell culture system will also differ from what is encountered in vivo, and we did not measure direct indicators of fibrinolysis (e.g., fibrin D-dimer). Furthermore, variable results among the different ancrod clinical trials may reflect differences in study design and patient population rather than generation of fibrin clot formation in the microvasculature, and there is no current evidence relating in vivo clot formation to poor outcome in ancrod clinical trials.

Despite the positive results in STAT, ancrod did not improve the outcome for patients with stroke in ESTAT or ASP. The variable clinical findings do not rule out the possibility that ancrod may have a role in a subset of patients with stroke with elevated levels of fibrinogen. Further clinical studies should analyze effects of ancrod on indices of fibrinolysis in patient subsets with differing stroke outcomes. Nevertheless, ancrod-induced microvascular thrombotic occlusion is a potential explanation for adverse outcomes and could explain the lack of consistent effects of ancrod in stroke treatment.

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Figure 3. In vitro effects of ancrod. Fibrinogen (A) and FPA (B) antigen concentrations were measured in media conditioned by HBMVEC after incubation with ancrod for 6 hours. HBMVEC incubated with control, ancrod, fibrinogen, and fibrinogen + ancrod for 6 hours (C). Six-hour endothelial conditioned media containing fibrinogen was isolated from HBMVEC and further incubated with ancrod for another 6 hours (D). Pooled results from 3 independent experiments: values represent mean; error bars represent standard error. *P<0.0001 vs fibrinogen (A and B); vs control, ancrod, and fibrinogen (C); or vs fibrinogen (D). FPA indicates fibrinopeptide A; HBMVEC, human brain microvascular endothelial cell.
Disclosures
None.

References
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